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The AMR-ARRAY: A modular bead array detecting β-lactam, (fluoro) quinolone, colistin, aminoglycoside and macrolide resistance determinants in Gram-negative bacteria

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ABSTRACT

The aim of this study was to develop a highly multiplexed bead array to detect genes and/or mutations frequently associated with resistance to antimicrobials of the β-lactam, (fluoro)quinolone, colistin, macrolide and aminoglycoside families in Enterobacteriaceae such as *Escherichia coli*, *Shigella* spp. and *Salmonella* spp. Ligase Chain Reaction and the Luminex® technology were combined in a 53-plex assay designed to target selected genetic markers with 3 internal controls. The AMR-ARRAY consistently detected resistance determinants as compared to phenotypically expressed resistance for 94.7% (856/904) of the assessed resistances. When compared to resistance profiles inferred from whole genome sequencing results, the AMR-ARRAY showed a selectivity and specificity of 99.3% and 100%, respectively. The strong features of the AMR-ARRAY are (i) its competitive cost, currently 18€/sample (ii) its wide analytical scope, currently 50 markers covering 5 antimicrobial families, (iii) its robust and user-friendly design consisting in a single-tube assay conducted in 4 successive steps (iv) its relatively short turnaround time, less than 8 h (v) its ability to detect allelic variability at critical SNPs (vi) its open access and easily upgradable design, with probes sequences, procedure and software source code freely available. The use of the AMR-ARRAY as a screening method in official antimicrobial resistance monitoring could improve the granularity of the collected data and pinpoint remarkable isolates harbouring unusual resistance determinants thereby enabling fit-for-purpose selection of isolates for Whole Genome analysis.

1. Introduction

Antimicrobial resistance (AMR) is a major animal and public health problem. Monitoring AMR is one of the important pillars of national action plans against AMR(Anonymous, 2019; WHO, 2021).

Currently, these monitorings are still mainly based on phenotypic analysis by standardised methods like disk diffusion or broth microdilution (EUCAST, 2020). However, molecular characterisation of resistance is valuable to identify the genetic mechanisms underlying resistance and thereby assess its transferability potential. Genetic testing allows the identification of the co- and cross-selection mechanisms of resistance, and there is little doubt that future monitorings will more and more incorporate the genetic dimension (European Union, 2020). Standardisation and harmonisation of the genetic characterisation of AMR is still under implementation and different approaches are still used depending on the countries and on the sector (ECDC, 2016; EFSA and ECDC, 2021). Using PCR to detect AMR resistance determinants is an option but requires to conduct several different assays to detect a large number of genes as well as Sanger sequencing to identify alleles (if needed). This approach is neither time- nor cost-effective. Different commercial kits allowing the detection of resistance genes are available but they are often limited in terms of target range and are expensive (a.o. Check-points, STRECK, GENESIG, vitro ("Check-Points,", 2021; "GENESIG - Antibiotic Resistance: blaGES,", 2021; "STRECK - Antibiotic

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Resistance Kits,", 2021; "vitro - master diagnostica: AMR direct flow chip,", 2021)). The gold standard methodology for identifying resistance determinants is whole genome sequencing (WGS). However, for the screening of large collections of bacteria, as in a monitoring context, fast and cheap alternative methods targeting the most circulating resistance genes can provide added value.

Here, we describe a liquid bead array based on the Ligase Chain Reaction (LCR) targeting 53 markers and allowing the simultaneous detection of the most frequent genes and/or mutations associated with resistance to the β-lactam, (fluoro)quinolone, colistin, macrolide (focus on azithromycin) and aminoglycoside families of antimicrobials in *Enterobacteriaceae* (*Escherichia coli, Shigella* spp., and *Salmonella* spp.). The performance of the AMR-ARRAY was evaluated on bacterial collections characterized phenotypically and genetically with reference methods.

2. Material and methods

2.1. Isolates collection

Indicator *E. coli* isolates from food-producing animals ($n = 251$) and from food ($n = 236$) were selected among isolates gathered through the Belgian monitoring programs conducted annually following the EU decisions 2003/99/EC and 2013/652/UE (European Union, 2003, 2013). Animal and food isolates were collected by laboratories approved by the Federal Agency for the Safety of the Food Chain (FASFC) and antimicrobial susceptibility testing's (AST) were performed by the National Reference Laboratory (NRL) AMR, Sciensano. Isolates were collected as described in the different reports available on the FASFC website (FASFC, 2020) during official AMR monitoring. Clinical isolates of *Salmonella* ($n = 64$) and *Shigella* spp. ($n = 97$) were retrieved from the collection of the Belgian National Reference Center of *Salmonella* and *Shigella*. Nine isolates from the External Quality Assurance System organised by the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) were also assayed. Isolates ($n = 648$) were selected as to representatively cover AMR diversity observed in Enterobacteriaceae of Belgian origin and were used to assess the analytical scope of the AMR-ARRAY.

Control isolates originated from the EURL-AR, from Sciensano bacterial collection or from other sources as listed in Table S1. The AMR genetic determinants of these control isolates were characterized beforehand by Sanger or whole genome sequencing (Table S1).

2.2. Antimicrobial susceptibility testing

Antimicrobial Minimum inhibitory concentrations (MICs) were determined in the framework of the Belgian AMR official monitoring by broth microdilution with EUVSEC and EUVSEC2 plates (Sensititre™, Thermo Fisher Scientific, Waltham, MA, USA) on food-producing animal and food isolates, interpreted according to the EUCAST epidemiological cut-offs of the related year (EUCAST, 2021) as explained in ad hoc reports (FASFC, 2020) and used to assign reference R/S phenotypes (Table S2). ESBL and AmpC phenotypes were assigned as recommended by the European Food Safety Authority (EFSA) (EFSA and ECDC, 2017).

2.3. DNA extraction

Genomic DNA was extracted using the DNeasy® Blood and Tissue kit according to the manufacturer instructions for Gram-negative bacteria (Qiagen, Hilden, Germany). DNA purity and concentration was assessed with the nanodrop 1000 (Isogen Life Science, Utrecht, The Netherlands).

Alternatively, few colonies were suspended in 400 µl of sterile milli-Q water and vortexed. Bacterial concentration was adjusted to reach an absorbance at 600 nm between 1 and 2 and a final volume of 400 µl was kept. Samples were incubated at 100 °C for 15 min and centrifuged at room temperature for 5 min at 10,000 rpm. The supernatant was collected and stored at −20 °C.

2.4. Padlock shaped Probes (PLPs) design

DNA sequence targets were selected according to the most often encountered resistance genes, alleles and/or mutations to the aforementioned antimicrobial families in Belgium, neighbouring countries and Europe. Probes were designed using sequences available in different publications or on GenBank with Bionumerics 6.6 (bioMérieux SA, Marcy-l'Étoile, France) similarly to previously described (Table 1) (Boland et al., 2018; Wattiau et al., 2011). Left and right arms were trimmed to reach a melting temperature (TM) about 55 °C and 60 °C, respectively, with a ΔTM around 5 °C. The free energy (ΔG) score of intramolecular folding of the resulting sequence was selected to never exceed −10 kcal.mol^{-1} as assessed with the MFOLD algorithm (Zuker, 2003). The resulting sequence was BLASTed to rule out non-specific annealing reactions (Altschul et al., 1990) Sequences of the universal primers "reverse" (cUR) and "forward" (UF, see Table 1) were merged as well as an anti-TAG sequence matching a given TAG sequence of the MagPlex-TAG™ microspheres (Luminex, Austin, Texas). Probes are schematically represented in linear form as follows: **right 5'arm** – **cUR** – **AA** – **UF** – **anti-TAG** – **3'arm** where "AA" is a di-deoxyadenosine linker). Probes were validated with reference strains used as positive and negative controls (Table S1). For single nucleotide polymorphism (SNP) targets, positive controls were derived from strains displaying the exact sequence targeted by the probe at the polymorphic nucleotide position and negative controls were derived from strains displaying another nucleotide at this position.

2.5. Ligase chain reaction assay

The molecular method developed for the detection of AMR determinants is a multiplex assay based on the ligation of PLPs annealed to a matching target followed by a common PCR amplification as described in Boland et al., 2018 (Boland et al., 2018) with minor modifications. Primers and probes used in this study are listed in Table 1. The LCR assay was conducted in three successive steps: the first step (ligation) was conducted in a 10 µl mixture containing 1 µl DNA (≥10 ng.µl^{-1}) extracted as described above, 2 U of *Pfu* DNA ligase (#600191–51, Agilent, Santa Clara, CA), 1 µl of *Pfu* DNA ligase buffer 10× (#600191–52, Agilent, Santa Clare, CA), and a specific final concentration of each PLP detailed in Table 1. Ligation was conducted in a thermal cycler. After 3 min at 95 °C, 25 cycles of 30 s at 95 °C followed by 5 min at 65 °C were performed, followed by a 2-min final denaturation at 98 °C. The second step (exonuclease treatment) started with the addition of 15 µl of exonuclease mixture consisting of 67 mM glycine-KOH, pH 9.4, 2.5 mM MgCl2, 50 µg.ml^{-1} bovine serum albumin (BSA) (#B0262S, New England BioLabs, Ipswich, Massachusetts, USA) and 0.0015 U λ exonuclease (#M262S, New England BioLabs, Ipswich, Massachusetts, USA). The resulting 25-µl sample was incubated at 37 °C for 45 min, followed by inactivation at 95 °C for 10 min. The third step, PCR amplification, started with the preparation of a mix of 50 µl of 2× ABsolute qPCR Mix (Thermo Scientific, Waltham, Massachusetts, USA), 2 µl of universal reverse (UR) primer concentrated at 2.5 µM and 2 µl 5'Cy3-labeled universal forward (UF) primer concentrated at 20 µM. Thereafter 50 µl of this mix were added to each product from the second step. After 10 min at 95 °C, 30 cycles of 45 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C were performed, followed by a 15-min final elongation at 72 °C and denaturation at 98 °C for 2 min. LCR products (amplicons issuing from the PCR amplification of ligated PLPs) were then stored at −20 °C. Three control isolates were used in each experiment: one *E. coli* NDM-1 as a positive control of the LCR and bead-array steps, one *Staphylococcus aureus* as a negative control of the "ENTERO" probe and *E. coli* ATCC25922 as a negative control of all probes targeting resistance genes. Each probe was verified with corresponding reference strains used as positive controls on each new batch of probe mix (Table S1).

Table 1
Probes and primers used for the LCR assay and identification of the corresponding Luminex® MagPlex-TAG™ microspheres.

Antibiotic family	Probe/primer name	Sequence[a]	MagPlex-TAG™ microspheres	PLP concentration (pM)	Aim	Source[b]
	TEM-ABL104E	**AGTACTCACCAGTCACAGAAAAGCATC**-cUR-AA-UF-TACTTCTTTACTACAATTTACAAC**TACACTATTCTCAGAATGACTTGGTTG**	MTAG-A015	400	Identification of wild type nucleotide at the ABL104 position in *bla*$_{TEM}$ (nucl = G, aa = E)	(Cohen Stuart et al., 2010)
	TEM-ABL104K	**AGTACTCACCAGTCACAGAAAAGCATC**-cUR-AA-UF-ACAAATATCTAACTACTATCACAA**TACACTATTCTCAGAATGACTTGGTTA**	MTAG-A039	200	Identification of the mutated SNP at the ABL104 position in *bla*$_{TEM}$ (nucl = A, aa = K)	(Cohen Stuart et al., 2010)
	TEM-ABL238G	**GTGAGCGTGGATCTCGCGG**-cUR-AA-UF-ATTCAATACTATCTAACACTTACT**TTTATTGCTGATAAATCTGGAGCCG**	MTAG-A038	400	Identification of wild type nucleotide of the ABL238 position in *bla*$_{TEM}$ (nucl = G, aa = G)	(Cohen Stuart et al., 2010)
	TEM-ABL238S	**GTGAGCGTGGATCTCGCG**-cUR-AA-UF-ACATCAAATTCTTTCAATATCTTC**TTTATTGCTGATAAATCTGGAGCCA**	MTAG-A055	200	Identification of mutated SNP of the ABL238 position in *bla*$_{TEM}$ (nucl = A, aa = S)	(Cohen Stuart et al., 2010)
	TEM-all	**RGCAACAATTAATAGACTGGATGGAGG**-cUR-AA-UF-TTTATCAAATTCTAATTCTCAA**CGAACTACTTACTCTAGCTTCCC**[d]	MTAG-A073	400	Detection of *bla*$_{TEM}$ family genes	(Huehn and Malorny, 2009)
	SHV-ABL238S	**GCRAGCGGGGTGCG**-cUR-AA-UF-CTTAACATTTAACTTCTATAACAC**CCGATAAGACCGGAGCTA**[d]	MTAG-A030	400	Identification of the mutated SNP of the ABL238 position in *bla*$_{SHV}$ (nucl = A, aa = S)	(Cohen Stuart et al., 2010)
	SVH-ABL240K	**AGCGGGGTGCGCG**-cUR-AA-UF-TCTCTTTAAACACATTCAACAATA**GATAAGACCGGAGCTRGCA**[d]	MTAG-A047	400	Identification of the mutated SNP of the ABL240 position in *bla*$_{SHV}$ (nucl = A, aa = K)	(Cohen Stuart et al., 2010)
	SHV-all	**GCCGRCAGCACGGA**-cUR-AA-UF-AATCTCTACAATTTCTCTCTAATA**GGCGATAAACCAGCCC**[d]	MTAG-A061	800	Detection of *bla*$_{SHV}$ family genes	Multiple alignment of sequences available in GenBank
β-lactams						
	CTX-M1 group	**TYCGGCAAGTTTTTGCTGTACGT**-cUR-AA-UF-TTAACAACTTATACAAACACAAA**CCGACTGCGGCTCTAA**[d]	MTAG-A053	400	Detection of *bla*$_{CTX\text{-}M\text{-}1}$ group	Multiple alignment of sequences available in GenBank
	CTX-M2 group	**GCGAAAATCGTAACCCACGGTTTC**-cUR-AA-UF-TTCTTCATTAACTTCTAATCTTACT**TTCTGGCTGCGGCR**[d]	MTAG-A052	200	Detection of *bla*$_{CTX\text{-}M2}$ group (*bla*$_{CTX\text{-}M74}$ and *bla*$_{CTX\text{-}M75}$ will not be detected)	(Park et al., 2006)
	CTX-M8–25 groups	**ACATCGTGGGTTGTCGGGGATA**-cUR-AA-UF-ACTTATTTCTTCACTACTATATCA**CAGGCWGGGCTACCC**[d]	MTAG-A034	200	Detection of *bla*$_{CTX\text{-}M8}$ and *bla*$_{CTX\text{-}M25}$ groups	Multiple alignment of sequences available in GenBank
	CTX-M9 group	**GCGGCGAGAATCATCGCC**-cUR-AA-UF-TTAATACAATTCTCTCTTTCTCTAT**RCGATGTGCTGGCTTCA**[d]	MTAG-A054	600	Detection of *bla*$_{CTX\text{-}M9}$ group (*bla*$_{CTX\text{-}M137}$ will not be detected)	(Briñas et al., 2005)
	OXA-1 like	**CAGAAGCATGGCTCGAAAGTAGCT**-cUR-AA-UF-ACACTTATCTTTCAATTCAATTAC**TAGGAGATAAAGAAAGAAACAACGGATTAA**	MTAG-A018	200	Detection of *bla*$_{oxa\text{-}1}$ like ESBLs	(Huehn and Malorny, 2009)
	OXA-10 like	**CACCAGTTTCTAGGCCGATAATTGC**-cUR-AA-UF-CAAATACATAATCTTACATTCAC**TGAAAAYCTGATGCTCATTCTTTATGA**[d]	MTAG-A013	200	Detection of *bla*$_{OXA\text{-}10}$ like	(Batchelor et al., 2008)
	OXA-2 like	**CACAACTATCGTRCCTTTGGCTTGA**-cUR-AA-UF-CATAATCAATTTCAACTTTCTACTTTAACTCTATA**TTGGCGTTCGTCTGC**[d]	MTAG-A012	200	Detection of *bla*$_{OXA\text{-}2}$ like	(Batchelor et al., 2008)
	ACC-1	**GCAGTGACCGTCAACGGT**-cUR-AA-UF-CATCTTCATATCAATTCTCTTATTTT**TATTCCCGGTATGTCGGTC**	MTAG-A035	200	Detection of *bla*$_{ACC\text{-}1}$, *bla*$_{ACC\text{-}4}$ and *bla*$_{ACC\text{-}5}$, but not *bla*$_{ACC\text{-}2}$ and *bla*$_{ACC\text{-}3}$	(van Hoek et al., 2005)
	CMY-1 group	**CTGTGGGGGGCCGTG**-cUR-AA-UF-ATCTCAATTACAATAACACACAAATACATA**ATGCAACAACGACAATCCWTC**[d]	MTAG-A067	200	Detection of *bla*$_{CMY\text{-}1}$ group	(Pai et al., 1999)
	CMY-2 group	**ACGCGTCCTGCARCCATTAAAACT**-cUR-AA-UF-TTTACAAATCTAATCACACTATAC**AAACGAAGAGGCAATGACCAG**[d]	MTAG-A078	200	Detection of *bla*$_{CMY\text{-}2}$ group (*bla*$_{CMY98}$, *bla*$_{CMY\text{-}101}$, *bla*$_{CMY\text{-}100}$, *bla*$_{CMY\text{-}74}$,*bla*$_{CMY\text{-}}$	(Batchelor et al., 2008)

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Table 1 (*continued*)

Antibiotic family	Probe/primer name	Sequence[a]	MagPlex-TAG™ microspheres	PLP concentration (pM)	Aim	Source[b]
					$_{93}$, bla_{CMY-82}, bla_{CMY-83}, bla_{CMY-70} will not be detected)	
	AMPC-chr-M18A[c]	**TCGTTACAATCTAACGCATCGCCAA**-cUR-AA-UF-ACACTCATTTAACACTATTTCATT**AGTTGTCACGCTGATTGGTA**	MTAG-A074	400	Identification of the mutated SNP at position −18 in the *E. coli* chromosomal bla_{ampC} gene	(Peter-Getzlaff et al., 2011)
	AMPC-chr-M42T[c]	**GATAGCAGCCAGACCGTAGAAAACCC**-cUR-AA-UF-CATAAATCTTCTCATTCTAACAAA**CAATCAGCGTGACAACTGTCAA**	MTAG-A075	200	Identification of the SNP at position −42 in the *E. coli* chromosomal bla_{ampC} gene	(Peter-Getzlaff et al., 2011)
	KPC-all	**CCCGGGTGTAGACGGC**-cUR-AA-UF-TACACAATATTCATCATAACTAAC**CTTGTCATCCTTGTTAGGCG**	MTAG-A045	200	Detection of bla_{KPC} family	(Poirel et al., 2011)
	NDM-all	**TCTGGTTTTCCGCCAGCTCG**-cUR-AA-UF-TTTCTTAATACATTACAACAT**AC**CAACGGTTTGGCGA	MTAG-A025	200	Detection of bla_{NDM} family	(Poirel et al., 2011)
	OXA-48 like	**ACCACGCCCAAATCGAGGG**-cUR-AA-UF-CTATCATTTATCTCTTTCTCAATTAT**CACTTAAAGACTTGGTGTTCATCCTTA**	MTAG-A072	200	Detection of bla_{OXA-48} and its variants and $bla_{OXA-199}$ (bla_{OXA-54} will not be detected)	(Poirel et al., 2011)
	VIM-general	**CATCACGGACAATGAGACCATTGGA**-cUR-AA-UF-AATTTCTTCTCTTTCTTTCACAATTAA**TGTATCAATCAAAAGCAACTCATCRC**[d]	MTAG-A014	200	Detection of bla_{VIM} family (bla_{VIM-7} will not be detected)	Multiple alignment of sequences available in GenBank
	GYRA-83-LEU[c]	**GGCGGTYTATGACACGATCGTC**-cUR-AA-UF-AATCAACACACAATAACATTCATA**CATCCCCATGGTGACTT**[d]	MTAG-A048	200	Identification of the mutated SNP in the aa 83 in the *gyrA* gene *(nucl = T, aa = L)*.	Multiple alignment of sequences available in GenBank
	GYRA-83-WT[c]	**GGCGGTYTATGACACGATCGTC**-cUR-AA-UF-CAATTTACATTTCACTTTCTTATC**CATCCCCATGGTGACTC**[d]	MTAG-A051	200	Identification of the WT SNP in the aa 83 in the *gyrA* gene *(nucl = C, aa = S)*	Multiple alignment of sequences available in GenBank
	PARC-80-ILE[c]	**TATCGCCGTGCGGATGGTATTTAC**-cUR-AA-UF-TTCAATTCAAATCAAACACATCAT**TCGCTTCATAACAGGCGA**	MTAG-A064	200	Identification of the mutated SNP in the aa 80 in the *parC* gene *(nucl = A, aa = I)*	Multiple alignment of sequences available in GenBank
(Fluoro) quinolones	PARC-80-WT[c]	**TATCGCCGTGCGGATGGTATTTACC**-cUR-AA-UF-TTAAACAATCTACTATTCAATCAC**TCGCTTCATAACAGGCGC**	MTAG-A046	200	Identification of the WT SNP in the aa 80 in the *parC* gene *(nucl = C, aa = S)*	Multiple alignment of sequences available in GenBank
	QNRA-all	**ATGAAACTGCAATCCTCGAAACTGGC**-cUR-AA-UF-ACTTACAATAACTACTAATACTCT**GGCGCCGCTTTCA**	MTAG-A057	200	Detection of plasmid borne genes *qnrA*	Multiple alignment of sequences available in GenBank
	QNRB**	**ATGGCATCTCTCAGCATTGCGC**-cUR-AA-UF-ATACTTTACAAACAAATAACACAC**CTGCCATTGATAAATCACAGCTTTTRAAA**[d]	MTAG-A019	200	Detection of plasmid borne genes *qnrB***	Multiple alignment of sequences available in GenBank
	QNRS all	**CAGGGTGATATCGAAGGTTGCCATTTT**-cUR-AA-UF-TCATCACTTTCTTTACTTTACATT**GACATTTATTAACTGCAAGTTCATTGAA**	MTAG-A044	200	Detection of plasmid borne genes *qnrS*	Multiple alignment of sequences

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Table 1 (*continued*)

Antibiotic family	Probe/primer name	Sequence[a]	MagPlex-TAG™ microspheres	PLP concentration (pM)	Aim	Source[b]
						available in GenBank
	MCR-1	**GTGGTGGCGTTCAGCAGTCATT**-cUR-AA-UF-TAACTTACACTTAACTATCATCTT**CTTGCGCTGATTTTACTGCCT**	MTAG-A027	200	Detection of *mcr-1* gene	Multiple alignment of sequences available in GenBank
	MCR-2	**ATTGGACTATTTAGCAGTCAGTATGCGAG**-cUR-AA-UF-CTTAAACTCTACTTACTTCTAATT**TGCTGTTGCTTGTGCCG**	MTAG-A056	200	Detection of *mcr-2* gene	Multiple alignment of sequences available in GenBank
	MCR-3	**AGCCATGGCCCAACCTACTACAAG**-cUR-AA-UF-CACTTAATTCATTCTAAATCTATC**GTTGGCTTCCACCTGATAGGT**	MTAG-A028	200	Detection of *mcr-3* gene	Multiple alignment of sequences available in GenBank
	MCR-4	**AGCTCATGCGGCACGGCC**-cUR-AA-UF-ACTACTTATTCTCAAACTCTAATA**GGCTGATTGCGTTTAACGATACT**	MTAG-A033	200	Detection of *mcr-4* gene	Multiple alignment of sequences available in GenBank
Colistin						
	MCR-5	**GTCGGGCTGTAAAGGCGTCTGT**-cUR-AA-UF-TACATTCAACACTCTTAAATCAAA**ATTCTCTGGCGCGATAACCA**	MTAG-A026	200	Detection of *mcr-5* gene	Multiple alignment of sequences available in GenBank
	MCR-6	**GTCTGATTCAGCGTGCGATGACT**-cUR-AA-UF-ACTTCTTCATTCTCTTATCAAATA**GGATTATCCGACATTGGGTAAGA**	MTAG-B068	200	Detection of *mcr-6* gene	Multiple alignment of sequences available in GenBank
	MCR-7	**CTAGGGGCGCCACTGC**-cUR-AA-UF-CAATAAACATTCTTTACATTCTCA**AGTAAGGTGAGGGCCGA**	MTAG-B058	200	Detection of *mcr-7** gene	Multiple alignment of sequences available in GenBank
	MCR-8	**CTGCGGAAGACAGTGGTGTGT**-cUR-AA-UF-CACATTTCTTACTTTAAACTTA**CATTCCCATCTGTTTTCTCTCTTACAA**	MTAG-B049	200	Detection of *mcr-8** gene	Multiple alignment of sequences available in GenBank
	23SrRNA-2059-WT	**AGACCCCGTGAACCTTTACTATAGCTT**-cUR-AA-UF-CTTATCTCTACACTTTACTTAAAT**CGCGGCAAGACGGAA**	MTAG-B059	200	Identification of wild-type nucleotide 2059 of the *23SrRNA* gene (loci rrl a,b,c, d,e,g,h)	Multiple alignment of sequences available in GenBank
Macrolides						
	23SrRNA-A2059G	**AGACCCCGTGAACCTTTACTATAGCT**-cUR-AA-UF-AAATAACTCACTATTTCACTTACA**GCGGCAAGACGGAG**	MTAG-B008	200	Identification of mutated nucleotide A2059G of the *23SrRNA* gene (loci rrl a, b,c,d,e,g,h)*	Multiple alignment of sequences available in GenBank
	ERMB		MTAG-B007	200	Detection of *ermB* gene	

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Table 1 (*continued*)

Antibiotic family	Probe/primer name	Sequence[a]	MagPlex-TAG™ microspheres	PLP concentration (pM)	Aim	Source[b]
		TATTCACCGAACACTAGGGTTGCTCT-cUR-AA-UF-TACACAAACAATCTTTCACAATTT**GATTCTACAAGCGTACCTTGGA**				Multiple alignment of sequences available in GenBank
	MPHA	**CTTGCAGCCCGACATGGG**-cUR-AA-UF-CACATCTAATACTTTATACAATTC**CCTCCCAACTGTACGCA**	MTAG-B009	200	Detection of *mph(A)* gene	Multiple alignment of sequences available in GenBank
	AAC(3)-II	**GCTCGGTCGCCATCGAGAA**-cUR-AA-UF-CTAAATCACATACTTAACAACAAA**GAAACTATAGCAAATGCTTACGTGAA**	MTAG-A063	200	Detection of *aac(3)-IIa,c,d,e,f* genes	Multiple alignment of sequences available in GenBank
	AAC(3)-IV	**GTGTCTCGGCACCCCATAGG**-cUR-AA-UF-TCAAACTCTCAATTCTTACTTAAT**CCATCCTGAAGAATGGTGCA**	MTAG-A021	200	Detection of *aac(3)-Iva* gene	Multiple alignment of sequences available in GenBank
	AAC(3)-VI	**CCTATCCGGGGTGTCGGC**-cUR-AA-UF-ATTAAACAACTCTTAACTACACAA**CGCGATCAACGAATTTATCCGAA**	MTAG-A036	600	Detection of *aac(3)*-VIa gene	Multiple alignment of sequences available in GenBank
Aminoglycosides	AAC(6′)-Ib all	**CTGGGCAAAGGCTTGGGAAC**-cUR-AA-UF-TACAACATCTCATTAACATATACA**ACTGGCGAATGCATCACAA**	MTAG-A037	200	Detection of *aac(6′)-Ib, Ib-cr* group	Multiple alignment of sequences available in GenBank
	ANT(2″)-Ia	**GCTGTTACAACGGACTGGCC**-cUR-AA-UF-TACTACTTCTATAACTCACTTAAA**CAGATGATCGCCTCCCA**	MTAG-A029	200	Detection of *ant(2″)-Ia* gene	Multiple alignment of sequences available in GenBank
	APH(3′)-VIa	**GGATGCMTCGGAGGAAACTGC**-cUR-AA-UF-AACTTTCTCTCTCTATTCTTATTT**TTTGTTGAACGTTGCCTAAGAGA**[d]	MTAG-A043	200	Detection of *aph(3′)*-VIa gene	Multiple alignment of sequences available in GenBank
	RMTB	**AAATACCGCGCCCTTTGCC**-cUR-AA-UF-TCTCATCTATCATACTAATTCTTT**CTCCATCCTGGCCTCAAAA**	MTAG-A076	200	Detection of *rmtB1,2,3,4* genes	Multiple alignment of sequences available in GenBank
Control PLPs	ENTERO	**ATGTTGGGTTAAGTCCCGCAACG**-cUR-AA-UF-CAAACAAACATTCAAATATCAATC**CGTCAGCTCGTGTTGTGAA**	MTAG-A022	20	internal positive control for *Enterobacteriaceae* (control of LCR and Luminex reactions, used for normalisation of Luminex results)	Multiple alignment of sequences available in GenBank
	INVA_1437	**TCAAGCGCGTTCCGCAACACATAG**-cUR-AA-UF-TAAACATACAAATACACATTTCA**GCCAGACAGTGGTAAAGCTCA**	MTAG-A062	200	Internal positive control for *Salmonella* spp.	(Lauri et al., 2011)
	UIDA_563	**CGTGGTGACGCATGTYGC**-cUR-AA-UF-TTTCTCATACTTTCAACTAATTT**CCTGGGTGGACGATATCAC**[d]	MTAG-A020	200	Internal positive control for *Escherichia coli/Shigella* spp.	Multiple alignment of

(*continued on next page*)

Table 1 (*continued*)

Antibiotic family	Probe/primer name	Sequence[a]	MagPlex-TAG™ microspheres	PLP concentration (pM)	Aim	Source[b]
						sequences available in GenBank
–	Background control bead	**No sequences**	MTAG-A077	–	Background control bead	–
	Universal Forward primer (UF)	GTAGACTGCGTACCAATTC	N/A	N/A	PLP PCR amplifcation	(Boland et al., 2018)
Primers for LCR step 3	Universal Reverse primer (UR)	GACGATGAGTCCTGAGTAA	N/A	N/A	PLP PCR amplifcation	(Wattiau et al., 2011)
	cUR	TTACTCAGGACTCATCGTC	N/A	N/A	N/A	(Wattiau et al., 2011)

Abbreviations: Aa = amino acid, ABL = ambler, nucl = nucleotide, PLP = padlock shaped probe, SNP = single nucleotide polymorphism

[a] Nucleotide sequence of primers and probes (from 5′ to 3′). Bold characters highlight the sequence targeted on the template DNA, underlined characters indicate the anti-TAG sequence complementary to the Luminex® MagPlex-TAG™microspheres with the bead ID indicated in the fourth column; normal characters indicate nucleotides added to reach a final set of probes with evenly distributed sizes ranging from 95 to 120 nucleotides. N/A means Not Applicable.

[b] *« Source » refers either to the selected marker reference or to the nucleotide sequences source.*

[c] *PLPs dedicated to E. coli and Shigella* spp.

[d] *IUPAC code was used for a nucleotide of these sequences.*

[*] Means that no control was available to test the PLP.

[**] QNRB PLP is not able to detect the following alleles: qnrB4, 11, 12, 22, 31, 34, 36, 37, 52, 54, 63, 67, 73.

2.6. Detection of the LCR products on a bead-array platform

LCR products were thawed and hybridised with a mix of 54 microspheres (including one microsphere used as negative hybridisation control) coated with capture probes whose nucleotide sequences are listed in Table 1. Before hybridisation, the bead mix was pelleted on a magnet and homogenised in a hybridisation buffer (0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0) at a concentration of 50 beads of each type per µl. The hybridisation mixture consisted of 25 µl of this bead mix and 37.5 µl of the final LCR product. After denaturation at 96 °C for 90 s, hybridisation was conducted at 37 °C for 30 min, immediately followed by three washes performed by pelleting the beads on a magnetic bead separation system (V&P Scientific, San Diego, CA) for 1 min, removing the supernatant by forceful inversion, suspending the beads in 75 µl of a second hybridisation buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0) and pipetting up and down. The plate was then incubated at 37 °C for 15 min in the Luminex® 200™ instrument and 50 µl of the final solution were analysed at this temperature. Fluorescence signals were measured on at least 100 beads of each bead type and Median Fluorescence Intensities (MFI) were automatically generated.

2.7. Data analysis

The fluorescence signal of the *Enterobacteriaceae* positive control probe “ENTERO” was used as an internal reference to normalise the MFI signals observed for each sample according to the formula (MFI probe/MFI ENTERO) X 100. Results expressed in normalised MFI (nMFI) were evaluated against positive and negative thresholds. These thresholds were determined as to ensure a minimal ratio of 2 between positive and negative nMFI values.

2.8. Whole genome sequencing analysis

Next Generation Sequencing (NGS) was performed on a selection of isolates (n = 139) from the 251 *E. coli* isolates from food-producing animals. This selection was made to cover a variety of resistance profiles and to resolve some discrepancies observed during the AMR-ARRAY development. NGS was performed with a MiSeq sequencing platform (Illumina, San Diego, CA). The Illumina Nextera XT DNA library preparation kit was used to prepare the sequencing libraries, followed by sequencing with a 250-nt paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Raw sequenced reads were trimmed with Trimmomatic v0.38.0 with default settings [24]. SPAdes v3.12.0 was used for read assembly using default settings (Bankevich et al., 2012). AMR genes occurrence was investigated using ResFinder 4.0 for *E. coli* with default settings (Bortolaia et al., 2020). Chromosomal mutations were analysed with PointFinder with default settings (Zankari et al., 2017). All sequencing data were submitted to SRA under BioProject PRJNA746728 https://dataview.ncbi.nlm.nih.gov/object/PRJNA746728?reviewer=81b13psdah7q4stvmmooq24a6j and PRJNA670414. The latter is part of a colistin resistance study and the same isolates were used in the current study (Timmermans et al., 2021). Reads were mapped with BWA-MEM using default settings and visualised with Tablet (version 1–21.02.08) (Milne et al., 2013).

2.9. Data comparison

Each susceptibility profile and each resistance-associated genetic sequence determined experimentally on the isolates in scope of the present study were compared to the AMR-ARRAY results. For each resistance in scope, if one or several PLPs tested positive for the associated marker, results were considered concordant.

3. Results

3.1. AMR-ARRAY development

The AMR-ARRAY is composed of a set of 53 PLPs (Table 1) all present in a single mix. It is able to detect resistance determinants to the following antibiotic families: β-lactams, (fluoro)quinolones, colistin, macrolides and aminoglycosides. *bla*$_{TEM}$ and *bla*$_{SHV}$ ESBLs are discriminated from non-ESBL *bla* genes by targeting mutations involved in amino acids modification at Ambler positions 104 and 238 for *bla*$_{TEM}$ and 238 and 240 for *bla*$_{SHV}$ (Table 1). *bla*$_{CTX-M}$ genes from groups 1, 2, 9 and 8/25 are differentially identified as well as the *bla*$_{OXA-1}$, *bla*$_{OXA-2}$ and *bla*$_{OXA-10}$ like genes (Table 1). Specific mutations were targeted in the promotor region of the chromosomic *ampC* gene at position −42 and −18 (Table 1). These mutations are responsible for the overexpression of the AmpC cephalosporinase (Caroff et al., 2000; Peter-Getzlaff et al., 2011; Guérin et al., 2021; Jacoby, 2009; Tracz et al., 2007). Additional probes were designed to identify the AmpC phenotype-causing variants *bla*$_{CMY-1}$, *bla*$_{CMY-2}$ and *bla*$_{ACC}$ (Table 1). Targeted genes and mutations involved in (fluoro)quinolones or macrolides resistance and genes conferring resistance to carbapenems, colistin and aminoglycosides are listed in Table 1. The AMR-ARRAY probe set is adaptable and probes can be removed or added depending on the purpose of the analysis. The all-inclusive reagent cost per sample is currently 18€ for 94 samples for the full set of 53 PLPs. All steps and turn-around time of the AMR-ARRAY are illustrated in Fig. S1.

3.2. Comparison of susceptibility profiles vs. AMR-ARRAY results

Two hundred and fifty one *E. coli* isolates from food-producing animals expressing antimicrobial resistance phenotypically documented in preliminary studies were analysed with the AMR-ARRAY (Table S2). ESBL and AmpC phenotypes but also resistance to quinolones, colistin, gentamicin and azithromycin were independently compared with AMR-ARRAY results. The aim of this comparison was to assess whether the AMR-ARRAY detected genetic resistance determinants in most of the isolates expressing a resistance profile for the corresponding antibiotics. AmpC phenotype comparison with AMR-ARRAY results showed the weakest concordance of 58.5% (24/41) while concordance with azithromycin resistance was higher (88.2%, 90/102). All other concordances were above 95% with 96.2% (178/185) of concordance for quinolones resistance and 98.2% (111/113) for gentamicin resistance. Finally, the highest scores were observed for colistin resistance ($n = 41$) and for the ESBL phenotype ($n = 171$) with 100% concordance for both (Table S2).

One hundred twenty-four food indicator *E. coli* isolates with antibiotic resistance demonstrated phenotypically (Table S3) were also analysed with the AMR-ARRAY. The isolates were analysed for their ESBL phenotype, quinolones resistance and/or colistin resistance. Resistance to quinolones showed a concordance of 93.3% (111/119) and a concordance of 98.3% (117/119) was observed for the ESBL profile while 100% of concordance was obtained for colistin resistance (5/5).

Finally, 9 isolates resistant to meropenem provided by the EURL-AR were also analysed with the AMR-ARRAY. For each isolate, the AMR-ARRAY detected a carbapenemase-encoding gene (Table S3).

Considered globally, AMR-ARRAY results were 94.7% concordant (856/904) with susceptibility profiles irrespective of the origin of the isolates.

3.3. Comparison of genetic resistance profiles vs. AMR-ARRAY results

One hundred twelve food *E. coli* isolates (Table S4), 64 clinical *Salmonella* isolates (Table S5) and 97 clinical *Shigella* isolates (Table S6) were analysed with the AMR-ARRAY to assess genetic results obtained through PCR and Sanger sequencing (Table S4) or other validated Luminex® xTAG® assays (Table S5 and S6). The AMR-ARRAY detected correctly all genes/SNPs responsible for ESBL or BL phenotypes and all but one SNPs involved in resistance to quinolones (Table S4-S5-S6). For all isolates taken together, 351/352 (99.7%) expected determinants were correctly identified by the AMR-ARRAY (Table S4-S5-S6). The discrepant quinolone resistance result was probably due to a mutation causing an amino acid modification in the *parC* gene at codon position 80 (*Ser* to *Ile*) as described in the "comparison of WGS data vs. AMR-ARRAY results" section. This results in a variant *parC* allele unable to anneal with the AMR-ARRAY probes PARC-80-WT and PARC-80-ILE. As a result, for this discrepant isolate, the two *parC* PLPs tested negatively.

3.4. Comparison of WGS data vs. AMR-ARRAY results

A subset ($n = 139$) of the 251 animal *E. coli* isolate collection was sequenced through WGS (identified by prefix "VAR" in Table S2). Selection was made to cover a various panel of susceptibility profiles and included isolates displaying discrepant profiles (experimentally demonstrated resistance not detected with the AMR-ARRAY and vice versa). Resfinder and Pointfinder algorithms (Bortolaia et al., 2020; Zankari et al., 2017) were used to map antimicrobial resistance genes / mutations in WGS sequence data and to infer predictive resistance profiles. When assessed with the AMR-ARRAY, 697/702 determinants (SNPs and genes) expected from WGS analysis were detected by the

Table 2
Genes and mutations observed in 139 isolates detected by WGS and AMR-ARRAY.

Antibiotics	Resistance genes	Detected number by WGS	Detected number by AMR-ARRAY
Beta-lactams	*bla*$_{TEM-1}$	80	80
	bla$_{TEM-52}$	6	6
	bla$_{TEM-199}$	1	1
	bla$_{TEM-135}$	1	1
	bla$_{TEM}$*	7	7
	bla$_{SHV-12}$	10	10
	bla$_{SHV-2}$	1	1
	bla$_{CTX-M-1}$	19	19
	bla$_{CTX-M-2}$	6	6
	bla$_{CTX-M-3}$	2	2
	bla$_{CTX-M-14}$	11	11
	bla$_{CTX-M-15}$	12	12
	bla$_{CTX-M-27}$	1	1
	bla$_{CTX-M-32}$	5	5
	bla$_{CTX-M-55}$	4	4
	bla$_{OXA-1}$	8	8
	bla$_{OXA-10}$	2	2
	bla$_{CMY-2}$	3	3
FQs	*qnrB19*	7	7
	qnrS1	18	18
Colistin	*mcr-1*	29	29
	mcr-2	1	1
	mcr-3	1	1
	mcr-4	8	8
	mcr-5	1	1
Aminoglycosides	*aac(3)-II*	34	34
	aac(3)-IV	12	12
	aac(3)-VI	6	6
	aac(6′)-Ib all	13	13
	ant(2″)-Ia	6	6
Macrolides	*ermB*	8	8
	Mph(A)	37	37
SNPs	*parC*** and *gyrA***	278	273[A]
	ampC n-18	49	49
	ampC n-42	15	15
Total		702	697
Percentage detected		99.3%	

Abbreviations: FQs = fluoroquinolones *Unidentified allele through WGS. **targeted SNPs, both WT and mutated.
[A] Discrepancies are caused by an untargeted mutation next to the targeted one (see text for details).

AMR-ARRAY (Table 2). Failure to detect the expected determinants was limited to 5 quinolone-resistant isolates harbouring a *parC* variant characterized by an identical point mutation in the *parC* gene sequence at codon position 80 (Ser to Ile), resulting in a variant *parC* allele unable to anneal with the AMR-ARRAY probes PARC-80-WT and PARC-80-ILE. The *parC* allelic variant in scope was neither detected by the WT nor by the mutant PLP probe. These AMR-ARRAY results do not allow to draw any conclusion with regards to fluoroquinolone resistance.

Finally, WGS analysis resolved discrepant results noticed when assessing AMR-ARRAY performance on isolates susceptible to azithromycin. *Mph(A)*, known to confer azithromycin resistance (Gomes et al., 2017), was found through both WGS and AMR-ARRAY analysis in such isolates considered susceptible according to current epidemiological cut-off (>16 mg.L^{-1}).

4. Discussion

The AMR-ARRAY is a user-friendly method with a cost per sample ranging, from 24.50€ for up to 12 samples, 20€ for up 30 samples and 18€ for 94 samples. It has a turnaround time of 8 h (Fig. S1). The method is able to detect nucleotide polymorphism at critical positions (SNPs) which is an advantage as compared to classical PCR and to the majority of the available commercial kits. Moreover, the assay consists in 53 detection probes that are pooled in a single tube which is, to the best of our knowledge, the largest multiplexed assay available for antimicrobial resistance characterisation.

4.1. AMR-ARRAY scope and performance

Compared to WGS results, the AMR-ARRAY showed a selectivity and specificity of 99.3% and 100%, respectively. In some rare instances, WGS results showed that mutations next to the targeted SNP were responsible for false negative AMR-ARRAY results (e.g., *parC* PLPs). The undetected *parC* allele was not included in the scope of the array because this was not described at the time the assay was designed. The nucleotide mutation in *parC* causing false-negative results was limited to 5/139 sequenced isolates. It accounted for all instances where no resistance marker was detected by the AMR-ARRAY while resistance to fluoroquinolones was experimentally demonstrated. Expanding the AMR –ARRAY scope to better accommodate *parC* allelic diversity would relieve such false negative results. Incidentally, discordant AMR-ARRAY results and susceptibility profiles should trigger resolution by WGS and possible discovery of new *parC* variants causing fluoroquinolone resistance.

When compared to antibiotic susceptibility profiles, the AMR-ARRAY provided highly concordant results (concordance with 94.7% of the phenotypes (856/904)). The lowest concordance was observed for isolates expressing the AmpC phenotype (58.5%, 24/41) and could be associated with several genetic backgrounds untargeted by the array: expression of the *DHA* gene (although never identified by WGS in this study in spite of 34 AmpC isolates sequenced), expression of efflux pumps or porin inhibition. Finally, the few azithromycin-resistant isolates not associated with known resistance genes ($n = 12$, 11.8%) could carry other untargeted resistance determinants including mutations or overexpression of efflux pumps (Gomes et al., 2017). Among these 12 isolates, 10 were sequenced by WGS and results confirmed the absence of *mph(A)*, *ermB* or mutation in the *23S rRNA* gene targeted by the AMR-ARRAY. Besides, the AMR-ARRAY detected the Mph(A) phosphotransferase gene in azithromycin-susceptible isolates ($n = 14$). Nine of these 14 isolates were sequenced and WGS confirmed the presence of *mph(A)*. Different studies reported that microorganisms carrying only *mph(A)* with no other macrolides resistance gene may display very different resistance levels (Gomes et al., 2017). In our study, 3/9 azithromycin-susceptible isolates harboured *mph(A)* only. The genetic determinants causing azithromycin resistance in these 10 isolates remains however unidentified. Such observations illustrate the usefulness of the AMR-ARRAY as a screening method to select atypical and/or unusual profiles that deserve whole-genome sequencing for further investigation.

The ability of this array to target SNPs allows to distinguish a range of ESBL alleles from non-ESBL alleles of some families of β-lactamases like bla_{TEM} and bla_{SHV} and to detect resistance-causing mutations in e.g., bla_{ampC}, *parC* or *gyrA*. This is an advantage of the AMR-ARRAY as compared to the majority of commercial kits and to PCR assays. Another advantage is that different types of DNA extraction methods are compatible with the system, although boiled DNA extracts lead to higher background noise (i.e. a higher baseline level of fluorescence intensity than with DNA extracted with the Qiagen extraction kit). PLP design, LCR, detection on Luminex ®200™ and data analysis are easy-to-apply methods making the AMR-ARRAY a user-friendly method. As it relies on common reagents and devices and as data analysis does not require any specific software, this method can be easily adapted to different laboratory configurations. Moreover, the availability of highly multiplexed xTAG® Luminex® bead sets allows to detect simultaneously a large range of AMR determinants in a single tube/sample. The AMR-ARRAY can target 53 different markers (internal controls included) in a single mix of probes. This high level of multiplexing is quite unique to our knowledge and could still be extended since a total of 80 xTAG® beads are available for Luminex®bead-array hybridisation platforms (Luminex, Austin, Texas). However, all beads are not compatible with all xMAP instruments ("Luminex - MagPlex-TAG™ Microspheres,", 2021). Except for the MCR-6, MCR-7 and MCR-8 PLPs and the macrolides PLPs which are not compatible with the MAGPIX® instrument, all other PLPs described here can be also detected on Lx200 and FLEXMAP 3D instruments.

4.2. The AMR-ARRAY: a modular screening method

One other advantage of the AMR-ARRAY is its modular nature. Indeed, only part of a probe set can be incorporated in the assay (e.g., MCR probes) to answer specific epidemiological questions. The AMR-ARRAY can be used as a screening method to monitor the presence of critical resistance determinants in bacterial isolates, to detect specific genetic profiles or to select the most interesting isolates to sequence. Furthermore, as new resistance determinants emerge and genetic variability increases, new PLPs can be designed and incorporated to an existing probe set without having to re-validate the whole assay. In our experience, adding new PLPs to an existing probe set does not generally alter assay performance in terms of sensitivity, specificity or reproducibility. AMR-ARRAY adaptations or updates can hence be engineered rapidly if the epidemiological situation or monitoring scope evolves over time.

In conclusion, the AMR-ARRAY is a method that can be used (i) to document susceptibility profiles by providing information on the genetic nature of the resistance, (ii) to study multiple resistance determinants (including mutations) at once (multiplexing of 53 different probes in a single mix) in a large collection of isolates and (iii) as a screening method to detect the most frequent resistance determinants and select which isolates should be sequenced in priority (e.g., isolates for which no AMR determinants are found with the array while resistance is demonstrated). In this regard, the usefulness of the array was demonstrated for 10 azithromycin-resistant and 15 AmpC isolates in which no known resistance marker could be identified neither by array screening nor with ResFinder or PointFinder. The genetic determinants causing azithromycin resistance in the 10 isolates are unknown so far but may be investigated in future work. The method is freely accessible (probes sequences, procedure, software for results interpretation) and is a cost-effective alternative to WGS for the detection of several antibiotic resistance genes when studying large collections of isolates, e.g., in a nation-wide monitoring context.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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